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INDUCTION OF LIMB DEFORMITIES IN CHICKEN EMBRYOS BY THALIDOMIDE[1]

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Thalidomide was first claimed by McBride (1) and Lenz (2) to be the cause of certain congenital malformations characterized by limb deformities in babies delivered by mothers who received this drug during early pregnancy. In mammals treated with thalidomide during pregnancy, the results were controversial. Deformities of limbs and other organs similar to those seen in human cases were obtained in the progeny of experimental rabbits (3-8), mice (4) and rats (9). On the other hand, negative or doubtful results were associated with the use of different breeds, strains, or colonies of these animals (3-8). These variations in susceptibility to thalidomide were attributed by some workers to genetical factors and/or differences in special enzyme systems (7, 10).

In chicken embryos treated with thalidomide before incubation, 5 or 6 days after incubation, the abnormalities were mostly confined in the head region (10), or in the beak only (11).

In our laboratory, we have been working on the teratogenic action of thalidomide in chicken embryos since the first case of thalidomide baby in Taiwan was discovered by Yang *et al.* (12). The chicken embryo was chosen for study because most basic embryological knowledge about this species is available, and because its developmental stages can be readily determined. Furthermore, any abnormalities produced can be attributed, without any ambiguity, to the direct action of the drug rather than to possible indirect effects mediated through maternal endocrine or metabolic mechanisms.

The present experiment was designed to treat chicken embryos with thalidomide within and beyond the time of the appearance of limb primodia. The immediate purpose was to explore whether the teratogenic action of this drug could be correlated with the developmental stages as suggested in human cases (13, 14).

MATERIALS AND METHODS

Eggs. Fertilized eggs of white Leghorns which had been maintained as a closed flock for years in a local farm were used. Fertility rate of the eggs used was 80-90%. Standard conditions of handling and incubation were followed. A total of 310 eggs were employed.

Thalidomide. Thalidomide (N-phthalyl-glutamic acid imide) used in the present experiment was the product of Dai Nippon Pharmaceutical Co., Ltd., Osaka, Japan, sold under the trade name of Isomin. Each tablet containing 25 mg of thalidomide was suspended in 5 ml of phosphate buffered saline at pH 7.2 and autoclaved before use.

Injection. For experimentals, groups of eggs were injected through the yolk sac on the 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11th days of incubation respectively with a single dose of 0.5 ml (2.5 mg equivalent) of the thalidomide suspension prewarmed to 37 C. Control eggs of 1, 2 and 3 days of incubation were injected with 0.5 ml of saline buffered at pH 7.2. Eggs older than 3 days of incubation were not used for controls because their limb buds were well formed (15). Rigid aseptic precautions were resorted to and no antibiotic was added, because certain antibiotics were reported to have caused deformities in animals (16).

Both the treated and control eggs were candled twice daily until hatching. The dead and

1 Outline of the experiment was read at the Annual Convention of Taiwan Medical Association on November 25, 1962.

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hatched chicks were examined for possible deformities of wings, legs, other skeletal structures and viscera, *etc.* Attention was paid also to 154 newly hatched chicks and 55 chicks followed up to 3 months for the possible occurrence of clinical signs such as cervical paralysis, convulsive movement and curly toe paralysis, *etc.*, reflecting folic acid, vitamin B_6 and riboflavin deficiencies respectively (17). This was done because some workers suggested that thalidomide might exert an inhibitory effect upon enzyme systems (18) or disturb the metabolism of vitamin B-complex (folic acid, B_6, B_2, *etc.*) (10, 19, 20, 21).

In the present study, no attempt was made to establish the dose-response relationship between thalidomide and its teratogenic effect on chicken embryos.

RESULTS

As shown in the table, injection of thalidomide within 48 hours of incubation produced limb deformities in 2 (3.63%) out of 55 embryos: unilateral amelia (entire absence of the left leg) in one embryo injected at 48 hours of incubation (*Figure 1*), and hemimelia of left wing characterized by shortened and small humerus with shortened tibia-tarsus-metatarsus and oligodactylia (missing 1st and 2nd toes) of the foot in another embryo injected at 24 hours of incubation (*Figure 2*). On the other hand, the sham-injected controls of the same age did not show any limb deformities. Limb deformities were not found in eggs treated with thalidomide beyond 72 hours of incubation either. Thus the drug-sensitive stage of limbs falls within the time of the appearance of limb primodia, about 50 hours after incubation (15).

Beak malformations were found in 5 embryos injected with thalidomide at 48 hours (3 with brachygnathia inferior), 6 days (1 with brachygnathia superior with acrania) and 9 days (1 with brachygnathia superior) of incubation respectively. However, 1 embryo sham-injected at 24 hours of incubation showed similar malformations (brachygnathia superior with anophthalmia) too.

In thalidomide treated group, and only in them, retarded embryos were seen in 2 out of 24 embryos injected at 24 hours of incubation, 2 out of 31 embryos injected at 48 hours of incubation, 1 out of 23 embryos injected on 3 days of incubation, 1 out of 12 embryos injected on 6 days of incubation and 1 out of 16 embryos injected on 9 days of incubation.

32.1% (26/81) of the eggs injected within 48 hours of incubation showed ceasation of development within 3 days after injection. However, comparable mumber, 28.2% (13/46), of control eggs also failed to develop when injected within 48 hours of incubation.

In the present study, we were not able to find signs of vitamin B-complex deficiencies in 154 newly hatched chicks and 55 chicks observed for 3 months after hatching.

DISCUSSION

In the present study, dysmelia closely similar to those frequently found in thalidomide babies was experimentally produced by injecting chicken embryos with thalidomide within 48 hours of incubation. No limb deformities were found in the embryos treated beyond 72 hours of incubation. The drug-sensitive stage falls within the time of the appearance of limb primodia. The result lends further support to the conjecture that the developmental stage is of critical importance in thalidomide teratogenesis as suggested in human cases (13, 14).

Since limb deformities were induced in developing chicken embryos free from maternal influences, the abnormalities observed can be ascribed to the direct teratogenic action of thalidomide upon the embryo. Maternal endocrine or metabolic mechanism might have played an indirect role (22, 23), which accordingly complicated the picture, in thalidomide-induced teratogenesis in other experimental animals, but definitely played no part in a self-existing chicken embryo.

The incidence rate of 3.63% (2/55) of dysmelia cases induced by thalidomide in chicken embryos treated at predicted sensitive stage of limb development was lower than that of humans (about 20%) (1, 2), New Zealand White rabbits (3, 6), and Sprague-Dawley rats (6.9%) (9). However, doubtful or negative teratogenic effect of thalidomide was found on Wister, Long Evans, August, and other colonies of Sprague-Dawley rats (4, 6) and silver grey rabbits (6). Furthermore, most of the malformations found by Kemper (10) and the only malformation found by Cameron (11) in chicken embryos were not in the limbs. Variations in susceptibility to thalidomide among species, breeds, and individuals within a species have been attributed by some workers to genetical or enzymic factors (7, 10), but the mechanism of teratogenic action of the drug remains unknown. The comparatively low incidence of limb deformities observed in this study appears to be, partly at least, the result of poor absorption of the drug *via* the yolk sac

TABLE I

The effects of thalidomide on chicken embryos injected at different developmental stages

Days of incubation when injected	Experimentals							Controls						
	No. injected	No. died within		No. hatched	Limb deformities	Deformities of the head region	Retarded embryo	No. injected	No. died within		No. hatched	Limb deformities	Deformities of the head region	Retarded embryo
		1 day	2-3 days						1 day	2-3 days				
1	31	3†	4†	10	1/24*	0/24	2/24	25	5†	1†	7	0/19	1/19	0/19
2	50	9†	10	22	1/31	3/31	2/31	21	1†	6	12	0/14	0/14	0/14
3	30	5	2	20	0/23	0/23	1/23	20	3	1	14	0/16	0/16	0/16
4	13	1	1	9	0/11	0/11	0/11							
5	14	2	0	8	0/12	0/12	0/12							
6	15	0	3	9	0/12	1/12	1/12							
7	18	1	1	13	0/16	0/16	0/16							
8	22	1	1	16	0/20	0/20	0/20							
9	16	0	0	15	0/16	1/16	1/16							
10	18	0	0	17	0/18	0/18	0/18							
11	16	0	0	15	0/16	0/16	0/16							

† Including 10-20% non-fertile eggs.

* Numerator is number of chicken embryos showing positive signs; denominator is number of embryos tested.

at the early embryonic stage. A localized mass of the injected drug, with inert base of the tablet, was frequently found in the yolk of eggs died within 3 to 4 days after incubation. The sticky yolk at this developmental stage might have impeded the free diffusion of drug to embryo. The occurrance of unilateral instead of bilateral limb deformities in this study seems to favor this view.

Beak malformation with or without acrania were found in 5 out of 165 chicken embryos injected with thalidomide within as late as 9 days after incubation. However, similar malformation of the beak with anophthalmia was found in 1 out of 49 sham-injected embryos within 3 days of incubation. Our previous experience of several such malformations among thousands of eggs used in other experiments further refrain ourselves from attributing the cause of beak malformations to thalidomide in this study, although the abnormalities of the head region were the major findings of Kemper and Cameron (10, 11).

Although it is hard to conclude whether thalidomide caused the ceasation of some early germs in this study, similar observations of the retardation of developing embryos apparently caused by this drug suggest that it might be the case.

The present experiment indicates that thalidomide, even if it is an inhibitor on development, is not a strong inhibitor for vitamins B_2, B_6, or folic acid. It seems unlikely that the deformities produced in this study were caused by vitamin B-complex deficiency, because the chicks hatched and observed for 3 months did not show recognizable signs of vitamin B-complex deficiencies. However, it must be emphasized that the eggs used were originated from hens fed with well balanced ration.

SUMMARY

Thalidomide (Isomin®), when injected into the yolk sac of embryonating eggs within 48 hours of incubation, produced amelia (absence of an entire leg), shortened tibia-tarsus-metatarsus with oligodactylia on a foot (absence of 1st and 2nd toes) and humeral hemimelia of a wing (absence of radius, ulna and phalanges). The limbs of the chicken embryos injected beyond 72 hours after incubation were not affected. The drug-sensitive stage falls within the time of the appearance of wing and limb primodia.

Retardation of embryos was seen in several chicken embryos injected within as late as 9 days of incubation.

In thalidomide treated groups, beak malformations (brachygnathia superior, brachygnathia inferior) with or without acrania were found when the drug was injected within as late as 9 days of incubation. However, similar beak malformation with anophthalmia was also found in 1 sham-injected embryo.

The possible mechanism of teratogenic effect of thalidomide was discussed.

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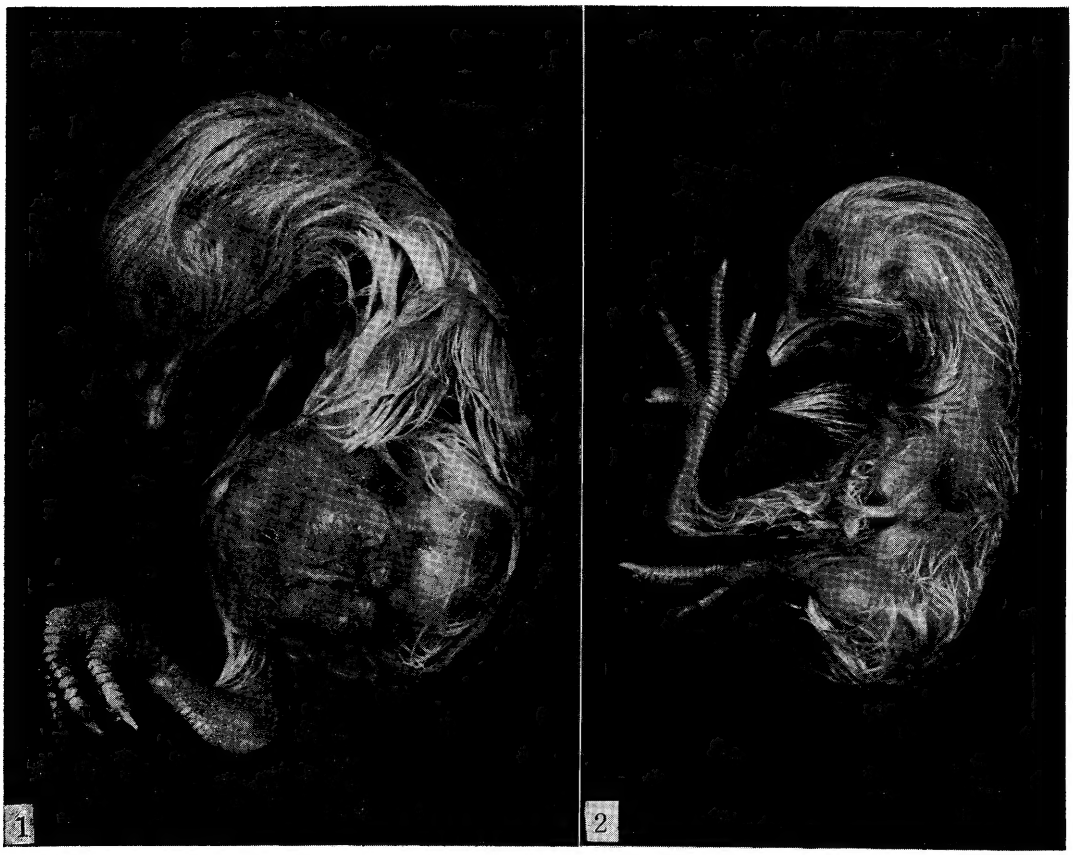

Fig. 1, Showing unilateral amelia caused by thalidomide given at 48 hours of incubation. The chick pipped but died on the 21st day of incubation (Original size).

Fig. 2, Showing shortened tibia-tarsus-metatarsus, oligodactylia missing 1st and 2nd toes on a foot and humeral hemimelia caused by thalidomide given at 24 hours of incubation. The embryo died on the 19th day of incubation (Original size).